Journal of Bioscience and Bioengineering
VOL. 120 No. 2, 231–237, 2015

ELSEVIER

www.elsevier.com/locate/jbiosc

# Hybrid organoids consisting of extracellular matrix gel particles and hepatocytes for transplantation

Jingjia Ye, Nana Shirakigawa, and Hiroyuki Ijima*

*Department of Chemical Engineering, Faculty of Engineering, Graduate School, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan*

Received 8 October 2014; accepted 5 January 2015
Available online 4 February 2015

**Hepatocyte transplantation is a potential therapy for treating various liver diseases. However, oxygen shortage leading to loss of hepatocyte function becomes a limitation following hepatocyte transplantation. To overcome this problem, we developed a hybrid organoid, consisting of growth factor (GF)-immobilizable gel particles combined with hepatocytes. Benefits of the hybrid organoid were evaluated in three groups: (i) hybrid organoid consisting of cells and GF-immobilizable gel particles (HG-C); (ii) hybrid organoid consisting of cells and gel particles (G-C); and (iii) cells suspended in collagen (C–C). We found liver-specific functions of HG-C were maintained longer than in the other conditions during *in vitro* culture. Furthermore, after transplantation, HG-C was effective in maintaining viability of transplanted hepatocytes and promoting angiogenesis around the hepatocytes. In summary, transplantation of HG-C is a potential method for future liver tissue engineering.**



[**Key words:** Immobilization; Tissue engineering; Organoid; Animal cell culture; Growth factor; Hepatocyte transplantation]

At present, liver transplantation is the only effective therapy for many patients suffering from liver diseases, including acute or chronic liver failure and liver cancer. However, this approach is limited by availability of donor organs. Consequently, techniques have been developed to transplant isolated human hepatocytes into recipient patients. Hepatocyte transplantation is a safer and cheaper procedure, in which a single liver could be utilized for several patients (1). However, hepatocytes are prone to losing their phenotype and biological function during *in vitro* culture (2). Furthermore, vascularization around the transplanted cells is often insufficient.

Cell–cell interactions between hepatocytes are essential for liver reconstruction and maintenance of liver function (3,4). However, hepatocytes have a relatively high intrinsic metabolic activity and are typically cultured at high density to maintain cell–cell interactions to enhance their differentiated function (5). The high density and metabolic activity of hepatocytes usually results in a lack of oxygen under *in vitro* culture (5).

In our study, we tried to establish a technology, termed 'hybrid organoid', using heparin-conjugated gel particles combined with hepatocytes. This technology allows structures to form with high local cell density and low total cell number, owing to the existence of gel particles (Fig. 1). In this way, cell–cell interactions are expected to be enhanced, accompanied by maintenance of oxygen consumption at low level. Additionally, growth factor (GF)-immobilizable gel is reported to enhance the formation of liver tissue and maintain liver-specific function of hepatocytes (6–8).

Heparin is demonstrated to interact with various GFs (9–13). Furthermore, heparin-based hydrogels are reported to be good scaffolds for hepatocyte culture, with the potential for transplantation. This approach is also an effective method for controlled release of GFs (10,14). Hepatocyte spheroid-embedded heparin-immobilized collagen gel was cultured and used for transplantation by Hou et al. (13). Cell viability and liver functions were improved after using heparin-immobilized collagen gel.

Survival of transplanted hepatocytes can only be maintained for long periods if a growth factor-cocktail is used during hepatocyte transplantation (15,16). Using this approach, a growth factor-cocktail, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), is secreted after hepatectomy treatment to improve angiogenesis (17–19). Furthermore, GFs like HGF and VEGF benefit the preservation of liver-specific functions (20,21). For transplantation in our study, rats were treated by hepatectomy. Using this approach, both angiogenesis and hepatocyte viability were improved. Moreover, the condition of hepatectomy was similar to that of liver failure.

The objective of our study was to investigate whether hybrid organoids can improve cell viability and liver function after transplantation.

## MATERIALS AND METHODS

**Reagents, animals and equipment** Reagents used in this study were as follows: collagen (Cellmatrix Type 1-A, Type 1-C) was purchased from Nitta Gelatin (Osaka, Japan) and gelatin (Procine skin type A; G2625) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Heparin sodium (100,000 units) and *N*-hydroxysuccinimide (NHS) were purchased from Wako Pure Chemicals (Osaka, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was purchased from Peptide Institute Inc. (Osaka, Japan). Dulbecco's modified Eagle's medium

* Corresponding author. Tel./fax: +81 92 802 2748.
*E-mail address:* ijima@chem-eng.kyushu-u.ac.jp (H. Ijima).

1389-1723/$ – see front matter 
http://dx.doi.org/10.1016/j.jbiosc.2015.01.004

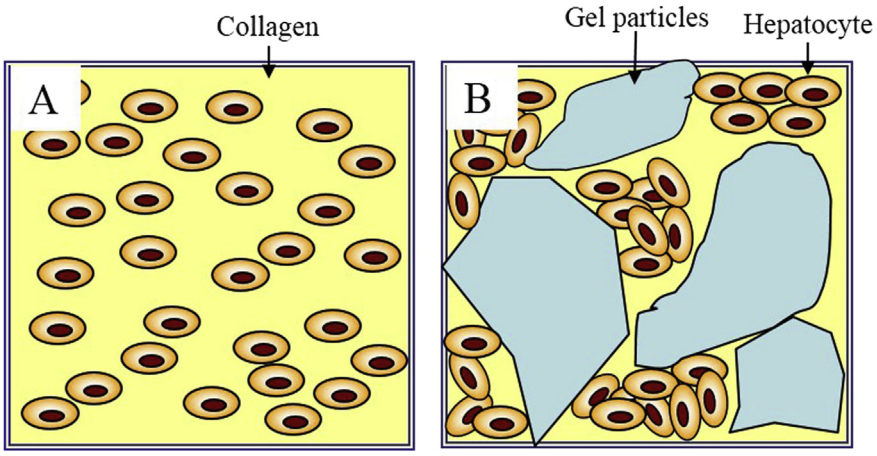


FIG. 1. Diagrammatic representation of a hybrid organoid. Identical total cell numbers are shown in both panels A and B. (A) Hepatocytes dispersed in the ECM. (B) Hybrid organoid consisting of gel particles and hepatocytes, with high local density and strong cell–cell contacts.

(DMEM), bovine pancreatic insulin, hydrocortisone and L-proline were purchased from Sigma. Epidermal growth factor (EGF) was purchased from Biomedical Technologies (Stoughton, MA, USA). The protein detector enzyme-linked immunoabsorbent assay kit, Horseradish peroxidase/2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic Acid Ammonium Salt) System, was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). Rat albumin standard and anti-rat albumin antibody were purchased from ICN Pharmaceuticals (Aurora, OH, USA). Polyurethane foam (PUF) was kindly donated by Inoac (Nagoya, Japan). Illustra RNA spin Mini RNA Isolation Kit was purchased from GE Healthcare (Berlin, Germany). High Capacity cDNA Reverse Transcription kit was purchased from Applied Biosystems (Courtaboeuf, France) Fluorescent DNA Quantitation Kit was purchased from Bio-Rad (CA, USA).

Male Wistar rats (6–8 weeks old) were purchased from Kyudo (Tosu, Japan). Cell morphologies were observed using a phase-contrast microscope (Eclipse TE 300, Nikon, Tokyo, Japan). The experimental protocol was reviewed and approved by the Ethics Committee on Animal Experiments of Kyushu University.

**Optimization of particle size** Heparin (5 mg) was activated with 0.24 mg EDC, containing 0.67 mg NHS, in 2.5 mL of 0.05 M 2-(*N*-morpholino) ethanesulfonic acid solution, for 10 min at 37°C. Gelatin sol (20%, 2.5 ml) was mixed with activated heparin solution (2.5 ml) at 37°C and allowed to cool down to gelation. This heparin-conjugated gel (5 ml) was then immersed in EDC (2 mg/ml)/NHS (8 mg/ml) solution for 3 h to permit cross-linking. Heparin-conjugated gelatin was obtained at a final concentration of 0.1 g/ml gelatin and 1 mg/ml heparin. Finally, the gel was crushed into small particles and separated according to particle size using 250-μm and 150-μm meshes to obtain three sizes of particles. Particles remaining on the 250-μm mesh were referred to as large-sized particles, particles remaining on the 150-μm mesh were referred to as medium-sized particles, and particles that passed through the 150-μm mesh were referred to as small-sized particles.

Hybrid organoids were formed by mixing 135 μl gel particles with hepatocytes ($3.58 \times 10^6$ cells) by centrifugal force (358 ×g, 2 min).

**Scaffold preparation for culture and transplantation** To keep the cell–cell connection *in vivo*, we used polyurethane foam (PUF) as a support. PUF is a well-characterized biocompatible macroporous scaffold in which cell aggregates can maintain their shape. Each pore of the PUF sponge-like structure is made up of smooth thin film with a thick skeleton (22). A block of PUF was cut into round disks (1.0-cm diameter × 2.0-mm thickness). Prior to use in the study, PUF disks were submerged in ultrapure water, de-aerated with a vacuum pump and sterilized by autoclaving in ultrapure water. Sterilized PUF disks were coated with Wistar rat serum (5× diluted) and frozen at −20°C overnight, prior to freeze-drying for 3 h. PUF disks were used to support shape maintenance of the samples during *in vitro* cell culture and transplantation.

**Sample preparation for culture and transplantation** Primary rat hepatocytes were obtained from 6- to 8-week-old male Wistar rats using a two-step collagenase perfusion method (23). Cell viabilities were greater than 85% using the trypan blue exclusion method.

Hybrid organoid samples, including hepatocytes and heparin-immobilized gelatin gel particle-embedded PUF (HG-C) and hepatocytes and gelatin gel particle-embedded PUF (G-C), were prepared as follows. Gelatin or heparin-conjugated gelatin gel particles (10%) were suspended in DMEM-based hormonally defined medium (DHDM) (22) overnight to remove excess EDC/NHS molecules, which are toxic to hepatocytes reducing their viability. Hepatocytes ($3.58 \times 10^6$ cells) and 135 μl of gel particles were suspended in DHDM and were inoculated onto the PUF disks by centrifugal force (358 ×g, 2 min).

Control samples, comprising hepatocytes and collagen gel-embedded PUF (C–C), were prepared as follows. Hepatocytes (at an inoculation density of $3.58 \times 10^6$ cells/well) were centrifuged in advance and the supernatants were removed. Hepatocyte pellets were mixed with 135 μl of 0.24% collagen solution (0.3% type I-A collagen, 10 × minimum essential medium and reconstruction buffer were mixed at a ratio of 8:1:1). The hepatocyte-suspended collagen solution was added to the PUF disks and solidified in 48-well plates at 37°C for 30 min.

Hybrid organoid (HG-C, G-C) and control (C–C) samples were then used in the following experiments.

**Cell culture** Hybrid organoid (HG-C and G-C) and control (C–C) samples were cultured in 48-well plates in 200 μl/well of DHDM containing EGF (50 μg/l), and were maintained at 37°C under 5% $CO_2$ in a humidified incubator. Medium was changed 4, 24 and 72 h after inoculation. Hepatocyte liver-specific function was determined by albumin secretion and ethoxyresorufin-*O*-deethylase (EROD) activity, on days 1 and 3 of culture. On day 3, cell viability was evaluated by DNA mass. After culture, samples were collected and stored at −80°C for one night. Freeze-thawing was repeated twice to enable crystallization to lead cell rupture. DNA-containing supernatants were then collected after 2 min centrifugation at 2000 rpm. DNA mass was measured using the Fluorescent DNA Quantitation Kit according to the manufacturer's instructions. Cell viability was calculated using the following equation (Eq. 1):

$$\text{Viability (\%)} = [\text{DNA mass in sample after culture/DNA mass before culture}] \times 100 \quad (1)$$

**Transplantation** Samples (HG-C, G-C and C–C) were transplanted into 70% partial hepatectomy (PH)-treated Wistar rats. Liver regeneration-related growth factors (HGF, EGF) are secreted after hepatectomy (24,25). Samples were therefore retrieved 7 d post-transplantation for subsequent analysis. Samples were soaked in formalin and sectioned for histological staining. Paraffin-embedded sections (4-μm thickness) were stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) stain. H&E stained sections were used to evaluate angiogenesis. We obtained approximately 100 random photographs for each sample group (HG-C, G-C, C–C). Vessel numbers and cross-sectional areas were measured using Image J software.

H&E stained sections were also used for evaluation of hepatocyte cell cluster size and cell number by counting the nuclei in the sections. Cell viability was calculated using the following equation (Eq. 2):

$$\text{Index of viability (\%)} = [\text{cell number (cells)/section area (mm}^2\text{)}] \times 100 \quad (2)$$

**Reverse transcription polymerase chain reaction** Gene expression levels of albumin and β-actin in the samples were evaluated after transplantation by reverse transcription polymerase chain reaction (RT-PCR). Total mRNA was extracted from samples using the RNA isolation kit according to the manufacturer's recommendations. Reverse transcription was performed with 0.45 μg total mRNA using the High Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions. cDNA synthesis was conducted at 37°C for 2 h and was terminated by the inactivation of reverse transcriptase after 5 min at 85°C.

Each PCR reaction was carried out using 0.45 ng/μl cDNA and 0.5 pmol/μl primers under the following conditions: pre-denaturation for 2 min at 95°C for one cycle, followed by amplification of cDNA for 30 cycles, which consisted of denaturation for 10 s at 98°C, annealing for 1 min at 52°C and extension for 30 s at 72°C. The following primer set was used: ALB, 5′–TTCAAAGCCTGGGCAGTAG–3′ (forward) and 5′–AGTAATCGGGGTGCCTTCTT–3′ (reverse); β-actin, 5′–CCCAGAGCA AGAGAGGCATC–3′ (forward) and 5′–GACCAGAGGCATACAGGGAC–3′ (reverse). After PCR, DNA was confirmed by agarose gel electrophoresis.